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The Isolation of Exotic Newcastle Disease (END) Virus from Nonpoultry Avian Species Associated with the Epidemic of END in Chickens in Southern California: 2002–2003

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SUMMARY. During the first 11 months of the 2002–2003 exotic Newcastle disease (END) epidemic in chickens in southern California, a total of 27,688 cloacal and tracheal (oropharyngeal) swab pools and/or tissue pools from 86 different avian species other than chickens and turkeys were submitted for Newcastle disease virus (NDV) isolation and characterization. Fifty-seven specimens (0.23%), representing 12 species of birds and 13 unspecified species, from a total of 24,409 accessions or submissions were positive for NDV. The NDV isolate was characterized as ENDV by real-time reverse transcription-polymerase chain reaction (RT-PCR). Of the 11,486 premises with other avian species, 1599 also had chickens. There were 1900 positive chicken samples from 164 premises, and 56 positive other avian species from 51 premises. Twelve premises had both positive chickens and positive other avian species. All positive other avian species were located on premises either on or within a 1 km radius of known infected premises. In this epidemic, premises with positive other avian species were significantly more likely to have chickens, and were significantly more likely to have positive chickens (OR = 3.7, $P < 0.0001$).

RESUMEN. Aislamiento de un virus exótico de la enfermedad de Newcastle a partir de especies aviares diferentes a aves de corral asociado con la epidemia de la enfermedad exótica de Newcastle en pollos y gallinas ocurrida en el Sur de California durante los años 2002–2003.

Durante los primeros once meses de la epidemia de la enfermedad de Newcastle ocurrida durante los años 2002–2003 en pollos y gallinas en el Sur de California, se recibieron un total de 27 688 grupos de hisopos y de tejidos cloacales y traqueales (de la orofaringe) obtenidos a partir de 86 especies aviares diferentes a aves de corral (pollos, gallinas y pavos) para el aislamiento y caracterización del virus de la enfermedad de Newcastle. De un total de 24 409 casos registrados en el laboratorio, un total de 57 (0.23%) casos representando a 12 especies de aves identificadas y a 13 especies no identificadas fueron positivos al virus de la enfermedad de Newcastle. Se caracterizó el virus de la enfermedad de Newcastle aislado como virus exótico mediante la prueba de la transcriptasa reversa-reacción en cadena por la polimerasa en tiempo real. De las 11 486 instalaciones con otras especies aviares diferentes a aves de corral, 1599 también alojaban pollos o gallinas. Se obtuvieron 1900 muestras positivas a partir de pollos y gallinas en 164 instalaciones, y 56 muestras positivas a partir de otras especies aviares diferentes a aves de corral en 51 instalaciones. Doce instalaciones tenían pollos y gallinas, al igual que otras especies aviares, positivas al virus exótico de Newcastle. La totalidad de especies aviares diferentes a aves de corral positivas al virus exótico de Newcastle se encontraban en instalaciones localizadas en un radio de hasta 1 kilómetro de distancia de instalaciones identificadas anteriormente como infectadas. En esta epidemia, las instalaciones con aves de otras especies aviares diferentes a aves de corral positivas al virus exótico de Newcastle tenían una probabilidad significativamente mayor a tener pollos y gallinas, y tenían una probabilidad significativamente mayor de tener pollos y gallinas positivas al virus exótico de la enfermedad de Newcastle (OR = 3.7, $P < 0.0001$).

Key words: NDV, END, swabs, nonpoultry species, real-time RT-PCR

Abbreviations: CA = chorioallantoic; END = exotic Newcastle disease; ENDV = exotic Newcastle disease virus; HI = hemagglutination inhibition; NDV = Newcastle disease virus; PPMV = pigeon paramyxovirus; RT-PCR = reverse transcription-polymerase chain reaction; VVND = velogenic viscerotropic Newcastle disease

Velogenic viscerotropic Newcastle disease (VVND), also known as exotic Newcastle disease (END) or Asiatic Newcastle disease, has been reported in parts of Asia, Africa, and eastern Europe since 1926 (6). In domestic nonvaccinated chickens, END is characterized by rapid dissemination and high mortality. The clinical signs include depression, anorexia, lethargy, respiratory distress, coughing, gasping, greenish watery diarrhea, and fever. The neurologic form of the disease is characterized by clonic spasms, muscular tremors, torticollis, opisthotonos, and paralysis of the legs and wings. The incubation of END ranges from 2–15 days with a mean of 5–6 days (2). The gross lesions, suggestive of but not pathognomonic for END, may include diphtheritic or hemorrhagic lesions in the larynx, trachea, proventriculus, small intestines (associated with the Peyer's patches), cecal tonsils, and cloacal mucosa (7). Exotic Newcastle disease was first diagnosed in the US in California in 1950 among chukars and pheasants imported from Hong Kong (1). In April of the same year, a great horned owl (*Bubo virginianus*) was diagnosed with exotic Newcastle disease from a zoological park in Columbus, Ohio

(9). Two decades later the disease was diagnosed in two unrelated cases in the United States: in August 1970 in a pet-shop bird in New York City, and in chickens in El Paso County, Texas. Subsequent to this, the disease was diagnosed in Massachusetts, New Mexico, Florida, California, Connecticut, Puerto Rico, Illinois, Arizona, and Florida (16). In New Mexico and Texas the disease was suspected of being introduced with infected fighting cocks from Puerto Rico (8). A catastrophic outbreak occurred in 1971–1973 in commercial flocks in southern California through the importation of infected parrots from South America. That outbreak spread to poultry in six counties (1). This strain of the disease has been described as indistinguishable from the first reported Newcastle disease strain isolated in 1926 (8). Many species of wild and captive birds are susceptible to END (5,14,21,22). Psittacines and other pet birds have been known to harbor the virus or have been implicated in the spread of END for the last several decades (10). Senne *et al.* reported that of the 2274 lots of pet birds offered for importation to the United States between 1973 and 1981, 6.5% (147 birds) were positive for END (16). However, the importation of pet birds through USDA quarantine stations has significantly dropped, and illegally imported birds, which are not tested for END, pose the major risk for the poultry industry (13). Since 1974, END has been found in nonpoultry birds in the United States every year (except 3 years) from domestic pet birds with no history of origin or that were suspected of being smuggled into the country (13). In May 1998, END was diagnosed in a flock of game chickens in Fresno, California (4), and again May 2002 in two ringneck parakeets in Redding, California (northern California). The ringneck parakeets were reported to have originated from a swap meet in southern California. In both instances the outbreaks were confined to the flocks, and the source could not be determined. More recently, END was diagnosed in backyard chickens in October 2002 and subsequently spread to commercial egg-laying flocks in December 2002 (Kinde, unpubl. data). This paper describes the epidemiology of END in nonpoultry species that were tested from inside and outside of the quarantine zone through the course of the 2002–2003 outbreak.

MATERIALS AND METHODS

Between October 2002 and August 2003, 27,688 specimens consisting of swabs and/or carcasses representing 86 species were submitted for exotic Newcastle disease virus (ENDV) testing to the San Bernardino Branch of the California Animal Health and Food Safety Laboratory System, University of California, School of Veterinary Medicine, Davis. Up to five swabs or carcasses were considered a sample. In the early days of the outbreak, necropsies were performed on carcasses submitted to the laboratory. Tissue pools of lung, trachea, and spleen, and pools of intestines were tested by virus isolation. During the course of the epidemic, cloacal and oropharyngeal swabs were also collected and tested by virus isolation. During the later half of the epidemic, tissue pools and swab pools were tested by real-time reverse transcription-polymerase chain reaction (RT-PCR).

Virus isolation. Tracheal, pharyngeal, and cloacal swabs were eluted in 3–5 ml of brain/heart infusion broth (BHI; Difco Laboratories, Detroit, MI) or Viral Transport Medium (VTM, CAHFS-Davis; containing 9.6 g Minimal Essential Medium, 20 ml 1M Hepes buffer, 3.57 g $NaHCO_3$, ACS grade, 250 mg gentamicin, 2500 μg amphotericin B in 1 liter triple distilled H_2O; Gibco, Grand Island, NY) which had been dispensed in 15 ml polypropylene centrifuge tubes. Swabs from up to five birds were pooled per tube. Ten percent tissue suspensions were prepared by placing small pieces (approximately 5 mm^3) of tissue in 15 ml polypropylene centrifuge tubes containing 3–5 ml of VTM, then frozen (−80 C) and thawed three times. The tubes containing tissue were then placed on a vortex mixer and agitated vigorously for 30 sec. Tubes containing swab fluids or processed tissue homogenates were centrifuged at 1500 × *g* for 10 min at 4 C. Two ml of supernatant were transferred to a 5 ml tube containing 1.3 ml of an antibiotic cocktail that resulted in a final concentration of 10,000 IU/ml of penicillin, 2000 IU/ml of streptomycin, 650 μg/ml of Kantrim, 1000 μg/ml Gentocin and 20 IU/ml of Mycostatin. The fluids were mixed thoroughly on a vortex mixer, then incubated at room temperature for 60 min. Each fluid was inoculated into the chorioallantoic (CA) sac of three 9-day-old embryonating specific pathogen free (SPF) chicken eggs, 0.2 ml per egg. Inoculated eggs were placed in a 37 C incubator and were candled for viability twice daily for 5 days. Eggs with viable embryos on day 5 postinoculation were discarded. Embryos that were dead >24 hours were chilled, the CA fluid was harvested for hemagglutination (HA) testing, and the positive hemagglutination CAF was further tested for hemagglutination inhibition (HI) using specific Newcastle disease virus antiserum (8,18). The positive HI samples identified as Newcastle disease virus (NDV) were further characterized by real-time (RT)-PCR with direct sequence analysis of the amplicon of the fusion protein cleavage site (Sharon Hietala, California Animal Health and Food Safety Laboratory, University of California, Davis, CA, pers. comm.). HA-negative fluids were inoculated into three embryonated eggs as above for a second passage before reporting a sample as negative for NDV.

Tracheal and oropharyngeal swabs from 7315 carcasses of nonpoultry species submitted for necropsy were collected during the 11-month period. Tracheal and cloacal swabs were obtained from the field by task force personnel in cases where carcasses were not submitted. In the early stages of the epidemic the isolates were characterized at the National Veterinary Services Laboratories, Ames, Iowa. Later in the course of the epidemic a rapid detection method using real-time RT-PCR was validated and implemented for testing oropharyngeal and cloacal swabs. However, virus isolation remained the primary testing method for psittacines that required movement permits and quarantine release. A positive case was defined as positive for NDV and subsequent characterization of the isolate as ENDV, or the detection of ENDV, was by real-time RT-PCR. ARCGIS 8.3 (ESRI, Redlands, CA) was used to characterize the geographic distribution of END-infected nonpoultry avian species in relation to game fowl, backyard chicken flocks, and commercial poultry premises within the southern California quarantine zone (Fig. 1).

Statistical methods. Chi-square tests (23) were used to measure the association between the disease status of "other avian species" with presence of chickens, and the association between the disease status of "other avian species" with the disease status of chickens. This analysis was limited to the backyard flocks from which "other avian species" were tested. A premise was considered to have chickens if test results were available for submissions from chickens.

RESULTS

None of the ENDV-positive nonpoultry species showed clinical signs or lesions except the pheasants. Specimens from 57 swabs or carcasses (tissue pool from five carcasses or five swabs) from a total of 24,409 accessions tested positive for NDV, with the isolate subsequently characterized as END virus by real-time RT-PCR (Table 1). The positive tissues or swabs represented 12 different species (one cockatiel, six doves, two ducks, two emus, three geese, one owl, four parakeets, two parrots, four peafowl, five pheasants, 12 pigeons, two quail, and 13 unspecified species; Table 1). All of the positive birds were collected inside the quarantine zone within 1 km of a known infected premise. Among the 51 premises was a small commercial pheasant farm ($n = 1600$). The five pheasants showed clinical signs (lethargy, increased mortality, diarrhea) and gross lesions (diphtheritic oropharyngitis, necrohemorrhagic enteritis, fibrinohemorrhagic enteritis) that were consistent with END. One of the 12 pigeons submitted was reportedly observed falling into a pond during sample collection on an infected premise. This pigeon had gross lesions

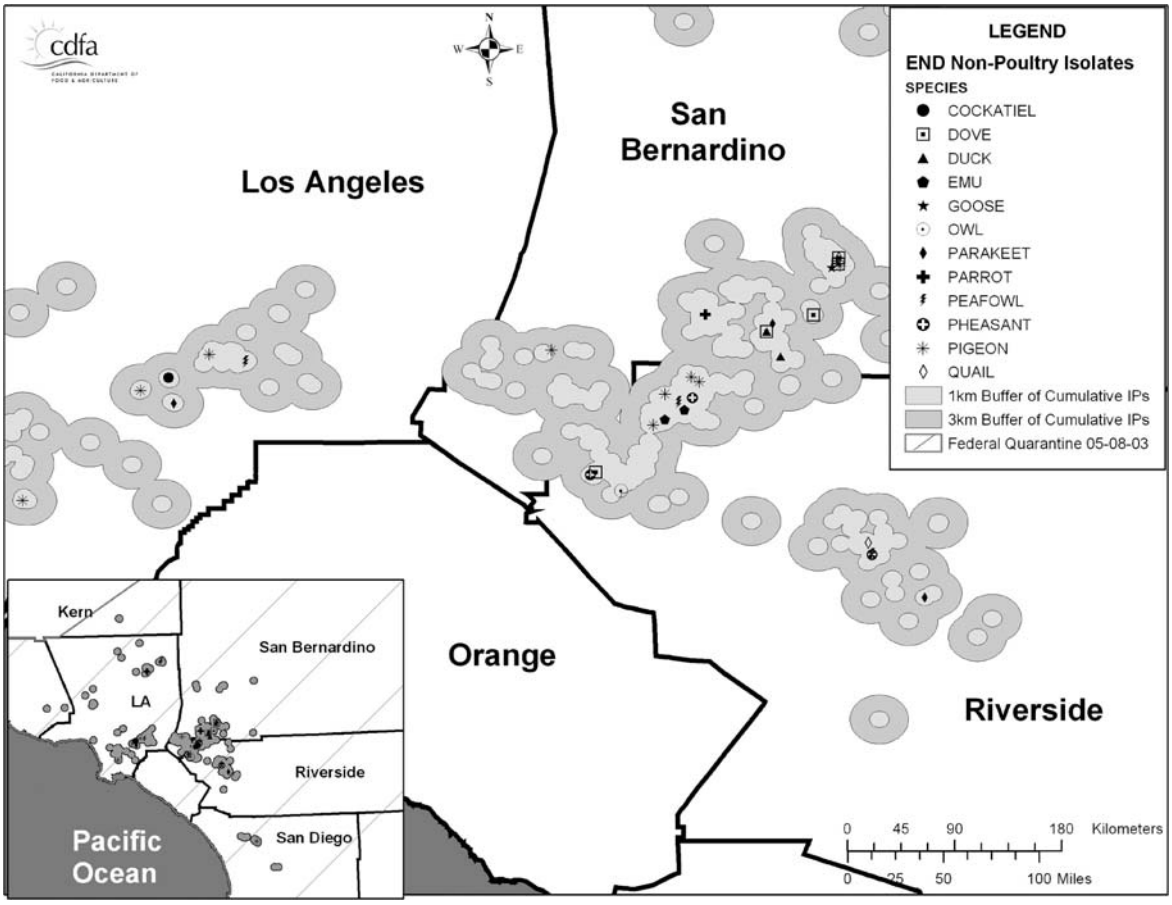

Fig. 1. Geographical distribution of nonpoultry positive-ENDV birds in the quarantine zone.

characterized by hemorrhagic proventriculitis and ventriculitis. Five other pigeons and one dove were positive for pigeon paramyxo virus.

Of the 11,486 premises with other avian species, 1599 also had chickens. There were 1900 positive chicken samples from 164 premises and 56 positive other avian species from 51 premises. Twelve premises had both positive chickens and positive other avian species. Thirty-seven percent (19/51) of the premises with positive other avian species had chickens, compared to 14% (1580/11,435) of premises with negative other avian species (OR = 3.7, $P < 0.0001$). For those 1599 premises with chickens and other avian species, 63% (12/19) of premises with positive other avian species had positive chickens, compared to 10% (152/1580) of premises with negative other avian species (OR = 16.1, $P < .0001$).

DISCUSSION

In previous END epidemics in California, exotic species of smuggled birds, particularly psittacines, have been known to play a major role in the introduction and dissemination of END, as these species may harbor and shed the virus without showing clinical signs (19). It is unclear how the disease in this epidemic was introduced to southern California; however, it has been determined that the virus has the same fusion protein cleavage site nucleotide sequence as the pet ringneck parakeet isolate from northern California in May of 2002, and as the virus which caused an END outbreak in Mexico in 2000 (15). It is interesting to note that during the VVND epidemic that occurred in southern California from 1971–1973, spread between chicken flocks was extensive and was due mainly to the movement of live birds and mechanical transport of the virus by humans, especially by vaccination and poultry service crews. Spread to exotic birds was from contact with infected imported stock. Spread to other species was most probably through contact with infected chickens (3,19). In the 2002–2003 epidemic, premises with positive other avian species were significantly more likely to have chickens, and were significantly more likely to have positive chickens. Nonpoultry species were most probably infected from contact with infected game fowl and perhaps did not play an important role in the dissemination of the disease. All of the infected premises were located within the quarantine zone and within a kilometer of another infected premise. In this epidemic, as in the 1972–1973 epidemic, free-flying birds did not play a major role. Three house sparrows and one crow were the only infected wild birds from which VVND virus was isolated in 1972–1973, and among semidomestic species, ducks, quail, chukars, pheasants, peafowl, pigeons, and doves were found to be infected (13), a remarkable similarity to the 2002–2003 outbreak. However, psittacines, pittas, and toucans accounted for 92% of the VVND virus isolations from exotic birds in the 1971–1973 outbreak (14). In the recent outbreak, only 0.12% (7/5681) of the psittacines were positive for ENDV. The nonexistence of USDA quarantine stations for imported exotic psittacines and absence of a surveillance program can explain such differences in the isolation rates of ENDV between the two epidemics for END prior to 1970. The 1971–1973 VVND epidemic in southern California raised public awareness, especially in the poultry industry and with regulatory officials, and as a result, active surveillance for VVND in pet birds was implemented and continued to exist through the mid-1980s. This active surveillance entailed sending test kits consisting of a viral transport medium and

Table 1. Nonpoultry species positive for END: ($n = 57/24{,}409$).[A]

Species	No. (%) Positive for ENDV
Psittacines	7/5681 (0.12) (4 parakeets, 2 parrots, 1 cockatiel)
Peafowl	4/593 (0.67)
Duck	2/3457 (0.06)
Pheasants	5/396 (1.26)
Pigeons[B]	12/5740 (0.21)
Dove[B]	6/4622 (0.13)
Emu	2/104 (1.92)
Goose	3/1744 (0.17)
Quail	2/822 (0.24)
Owl	1/7 (14.29)
Unspecified species:	13/1243 (1.05)

[A]Species that tested negative for ENDV: American goldfinch-1, American kestrel-1, blue jay-3, brown-headed cowbird-1, canary-426, cattle egret-1, chukar-74, Cooper's hawk-2, cormorant-2, Cornish game hens-2, cowbird-1, crow-57, great horned owl-1, guinea fowl-244, hawk-14, hummingbird-4, laysan teal-6, mallard-5, mixed-1317, mockingbird-12, moulard duck-1, myna-2, nene goose-1, northern jacana-1, ostrich-178, passerine-392, peacock-3, peacock (peahen)-84; pelican-2, penguin-4, raven-2, red-tailed hawk-1, ringneck dove-1, ringneck pheasant-2, rock dove-1, seagull-9, snow goose-3, sparrow-36, starling-5, swan-8, tern-1, turaco-1, turkey vulture-1, whistling duck-1, wild turkey-3, wood duck-1, woodpecker-1, wren-2, yellow-rumped warbler-1.

[B]PPMV was isolated from 5 other pigeons and 1 dove.

chlamydial medium along with a submission form to small animal practitioners throughout California with return postage and the address of the laboratory. As an incentive, the practitioners were provided with free testing for chlamydia when sending the Newcastle disease sample. Between the mid-1970s and the 1980s, ENDV was isolated over two dozen times using this surveillance system (Kinde, unpubl. data).

Pigeon paramyxovirus (PPMV) was isolated from five of 5740 pigeon samples and one of 4622 doves. This low incidence of PPMV is not surprising, as most pigeon owners are routinely vaccinating for the disease and the clinical disease is rarely seen in the California Animal Health and Food Safety Laboratory. No other virus was isolated from the nonpoultry species specimens.

Birds that are illegally smuggled into the United States are not quarantined and tested by the U.S. Department of Agriculture, and therefore may harbor the END virus without outward signs of the disease. Once introduced, the disease can spread quickly to backyard and commercial poultry and may have serious economic consequences. Estimates of the losses if END is uncontrolled in the United States range from $200 to $800 million in the first year (11,12,14,17). The 1971 epidemic of END in southern California spread to domestic poultry and was eradicated after 3 years at a cost of $56 million (12), ($243 million in 2004 dollars). In contrast, the current END outbreak was eradicated in one year at a cost of $167 million (20).

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